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Very rapid clearance after a joint bleed in the canine knee cannot prevent adverse effects on cartilage and synovial tissue

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Summary

Objective: Joint bleeding leads to joint destruction. *In vitro* exposure of human and canine cartilage to blood results in long-lasting severe adverse changes in cartilage. An *in vivo* joint haemorrhage in the canine knee joint demonstrates similar adverse effects although significantly less outspoken. As a possible explanation for this discrepancy, we studied the clearance rate of blood from the canine knee joints.

Methods: Blood was injected into the knee joint of Beagle dogs either 48 h, 24 h or 15 min before termination. The amount of red blood cells (RBC) and white blood cells (WBCs) present in the joint cavity was determined. Chondrocyte activity and cartilage matrix integrity as well as cartilage destructive activity of synovial tissue were determined biochemically. Additionally, synovial tissue was analyzed by use of histochemistry.

Results: The amount of blood was decreased to <5% within 48 h. Within this time period the cartilage was negatively affected and the synovial tissue showed cartilage destructive activity. Evaluation of the synovial tissue 15 min post-injection revealed countless numbers of intact RBC that were almost completely disappeared after 48 h without significant recruitment of macrophages.

Conclusions: Blood is cleared very rapidly from the canine knee joint, but already has adverse effects on both cartilage and synovial tissue within that short time span. This rapid clearance can play a role in the discrepancy between long-term *in vitro* and *in vivo* effects of blood-induced joint damage since more than 10% v/v blood present for at least 48 h is needed to induce long-term adverse effects *in vitro*.

Key words: Cartilage, haemarthrosis, Clearance, Arthropathy, Synovial tissue.

Introduction

Recurrent joint bleeding leads to damage of articular cartilage and eventually to destruction of the joint[1–4]. In case of repeated joint bleeds as in the clotting disorder haemophilia, this is a generally observed phenomenon, but it can also occur after joint trauma. Insight into the pathogenetic mechanism of blood-induced arthropathy has evolved from various *in vitro* and animal *in vivo* studies. It is shown that blood has a direct effect on cartilage, which is caused by the combination of mononuclear cells (MNC) and red blood cells (RBC) as present in whole blood[5]. *Via* several steps apoptosis of chondrocytes is induced[6], leading to serious disturbance of cartilage matrix turnover. Furthermore, joint bleeding has an effect on the synovial tissue, which initially is expressed by hypertrophy of the synoviocytes, hypervascularization[3], even increasing the risk of new bleeds, and by inflammatory changes[7,8]. The inflammatory changes also contribute to the cartilage damage, by production of tissue-destructive enzymes and cytokines[9]. These processes ultimately lead to joint destruction.

In vitro studies revealed that an exposure of 50% volume/volume (v/v) blood for 4 days leads to long-lasting (up to 10 weeks) severe inhibition (on average >70%) of cartilage matrix proteoglycan synthesis for both human and canine full thickness cartilage explants[10]. This blood load was considered to be an underestimation of the blood load during a joint haemorrhage as seen in humans. Traumatic joint bleeds or joint bleeds due to haemophilia are believed, based on clinical practice, to reach a blood concentration far above 50% v/v and if the blood is not aspirated, it usually takes several days before the effusion subsides. Later *in vitro* studies revealed a threshold of 10% v/v blood for 2 days to be a minimum blood load to induce long-lasting disturbance of cartilage matrix turnover[11]. Lower concentrations or shorter exposure times led to less severe acute changes that were reversible.

In *in vivo* canine experiments two injections of blood in the knee, with 2 days in between, were assumed to mimic a blood load of 4 days of at least 50% v/v. This resulted in direct harmful effects as demonstrated by a decrease in proteoglycan synthesis, an increase in proteoglycan release and even a decrease in proteoglycan content and enhanced collagen damage[12]. These effects, however, were significantly less severe than those *in vitro*. Even

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repeated (up to 12) intra-articular blood injections with intervals of 3–4 days did not result in the extreme effects as observed *in vitro*. Even more surprisingly, in later studies the effects appeared to be largely reversible within a follow-up of 10 weeks[10]. However, when the dogs were forced to load their affected joint, more permanent adverse changes occurred[13], from which it was concluded that one should take care with loading of a joint after an intra-articular haemorrhage.

Overall evaluation led to the conclusion that there is a significant discrepancy between the severities of the harmful effect of blood on cartilage *in vivo* when compared to the effect *in vitro*. An explanation could be a much more rapid clearance of blood from the canine knee joint as originally anticipated on, resulting in a blood load below threshold values that induce long-lasting effects. Therefore, the present study was undertaken to study the clearance rate of blood from the canine knee joint in relation to its harmful effect.

Materials and methods

EXPERIMENTAL DESIGN

Six skeletally mature female Beagle dogs (mean age 8.2 ± 1.2 years) were obtained from the University Utrecht Central Animal Facilities. They were housed in groups of two and were fed a standard commercial diet with water *ad libitum*. The University Animal Experiments Ethical Committee gave approval for this study. Approximately 4 ml freshly collected autologous blood was injected into the knee while the dog was under short-term anaesthesia (Domitor™/AntiSedan™). This was done either 48 h (dogs 1–3; left), 24 h (dogs 4–6; left) or 15 min (dogs 4–6; right) before the scheduled sacrifice of the dogs. The control joint (dogs 1–3; right) was not injected at all in order to prevent possible iatrogenic bleeding. Dogs were euthanized by intravenous injection of 10 ml Euthesate™ (200 mg/ml). The hind limbs were amputated and, upon minor arthrotomy, samples of synovial fluid were taken. Thereafter, synovial tissue from the suprapatellar pouch and cartilage of the femoral condyles and tibial plateau of the knee joints were collected. All these latter procedures were carried out under laminar flow conditions immediately after the animals were euthanized. The amounts of RBC and white blood cells (WBC) present in the joint fluid were determined immediately, by automated cell counting (Coulter Counter, Beckman).

Cartilage was cut as thick as possible, taking care to exclude the underlying bone, and was subsequently cut into square pieces, weighed aseptically (5.8 ± 0.3 mg) and incubated individually in 96-well round-bottomed microtitre plates in 200 μl culture medium per well. For the determination of the *ex vivo* proteoglycan synthesis rate as a measure of the chondrocyte matrix synthesis, the rate of sulphate incorporation was determined. To this end, $Na_2^{35}SO_4$ (DuPont, NEX-041-H, carrier free) was added in 10 μl aliquots of 148 kBq per well. After 4 h pulse-labelling of the sulphated glycosaminoglycans (GAGs), the cartilage samples were washed twice in ice cold Phosphate Buffered Saline (PBS) and stored at −20°C. Thawed samples were digested with papain (Sigma P3125) as described previously[11].

For the determination of the proteoglycan release, cartilage explants were pre-cultured for 1 day to remove detached proteoglycans, resulting from cutting the cartilage samples. After this pre-culture, cartilage explants were put in fresh culture medium and cultured for 3 days after which the amount of proteoglycans released in this 3-day culture period were determined as described below.

The synovial tissue was cut into five pieces, of which three pieces were fixed in 4% phosphate-buffered formalin, for histochemistry as described below and two pieces were cultured for 4 days in 5 ml culture medium. After culture, the supernatants were harvested and rendered cell free by centrifugation ($1000\times g$, 10 min). The two supernatants of the synovial tissue samples of the same joint were pooled. These pooled supernatants were added to cultures of homologous (Beagle) cartilage from the hip joint, after 1 day of pre-culture of the cartilage, in 5% and 50% v/v concentration (diluted in culture medium). The change in proteoglycan-synthesis rate (5% cultures), -release (50% cultures) and -content (50% cultures) of the cartilage exposed for 4 days to the synovial tissue culture supernatants compared to control cartilage (without the addition of supernatants) was considered as a measure for the catabolic (tissue-destructive) properties of that synovial tissue sample.

Culture medium of both cartilage and synovial tissue cultures consisted of Dulbecco's modified Eagle's medium (DMEM), supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin sulphate (100 μg/ml), ascorbic acid (85 μM) and 10% heat-inactivated Beagle serum. Cultures were performed in a tissue incubator under 5% CO_2 in air, at 37°C, and 95% humidity.

CARTILAGE ANALYSIS

For the proteoglycan synthesis rate, the GAGs in the cartilage tissue digest were precipitated with 0.3 M hexadecylpyridinium chloride monohydrate (CPC, Sigma; C9002). The precipitate was dissolved in 3 M NaCl and the amount of radioactivity in the sample measured by liquid scintillation analysis. The rate of sulphate incorporation was normalized to the specific activity of the medium, labelling time and wet weight of the cartilage and expressed as nmoles of sulphate incorporated per hour per gram wet weight of the cartilage tissue (nmol/h g).

The proteoglycan content of the cartilage tissue digest was determined by the GAG content of the digest. GAGs were stained and precipitated with Alcian Blue[14,15] (Sigma A5268). Alcian Blue staining was determined by the change in absorbance at 620 nm. Chondroitin sulphate (Sigma C4383) was used as a reference. The proteoglycan content was expressed in mg GAG per gram wet weight of the cartilage explants (mg/g).

The DNA content of cartilage tissue digests was determined as a measure of the cellularity of the cartilage samples using the fluorescent dye Hoechst 33258 (Calbiochem 382061). Calf thymus DNA (Sigma D4764) was used as a reference. DNA content is expressed as milligram DNA per gram wet weight of cartilage (mg/g). In all conditions, there was no change in DNA content, viz. the cellularity, of the cartilage due to blood exposure.

Proteoglycan release was determined by the loss of GAGs in the culture medium. GAGs were stained and precipitated with Alcian Blue as described above. The proteoglycan release was calculated as mg GAG released per gram wet weight of the cartilage (mg/g).

SYNOVIAL TISSUE HISTOLOGY

For histochemistry, samples of synovial tissue, fixed in 4% phosphate-buffered formalin, were embedded in paraffin-wax after standard processing. Deparaffined sections were stained with either haematoxylin–eosin (HE) to quantify inflammation and the amount of RBC, with MAC387 (AbD Serotec MCA874G; human with canine cross-reactivity) for macrophages/monocytes or with Perls' Prussian Blue for iron deposits. Each specimen was analyzed by two independent observers who were blinded to the source of the samples. The severity of the inflammation was scored using the slightly modified[16] criteria described by Goldenberg and Cohen[17] for HE-sections. The presence of RBC was scored in the HE-sections as follows: 0: no RBC; 1: single RBC throughout the tissue; 2: small clusters of RBC; 3: large clusters of RBC. In the sections stained with MAC387 or with Perls' Prussian Blue the amount of positive cells per mm^2 were determined.

VERIFICATION EXPERIMENT

Two observations from this study were rather astonishing: the rapid clearance of RBC from the joint and the presence of huge amounts of intact RBC in the synovial tissue after 15 min. Therefore, the study was repeated for these two parameters. An identical experiment was performed (six dogs aged 9.8 ± 0.2 months) and both RBC and WBC count in the synovial fluid was performed as well as histochemistry of the synovial tissue.

CALCULATIONS AND STATISTICAL ANALYSIS

For the direct effects on cartilage, parameters were determined in six explants taken from fixed locations from the femoral condyles as well as in six from the tibial plateau. Because for all parameters, no differences in effect were found for the condyles and the plateau, the mean of the 12 samples was taken as representative for that particular knee.

For the cartilage destructive properties of the synovial tissue, each pooled supernatant was added to 8–10 cartilage explants. The mean of these 8–10 explants was taken as a representative for that supernatant.

The data were analyzed using a parametric test for unrelated samples (independent samples *T* test), using SPSS 12.0.1 software. Data are presented as means ± S.E.M. of three joints for each time point and parameter. Asterisks indicate a P value ≤ 0.05.

Results

CLEARANCE

Fifteen minutes after injection of autologous blood, the concentration of RBC in the synovial fluid was 5.7×10^{12}/l [Fig. 1(a)]. This number is within the normal range of RBC in whole blood of Beagles[18], suggesting that the joint cavity was almost completely filled with blood and that the volume of synovial fluid originally present was negligible. Twenty-four hours after the injection of blood, the amount of RBC was decreased to 1.6×10^{12}/l, and 48 h post-injection this amount was further decreased to 0.2×10^{12}/l, i.e., a

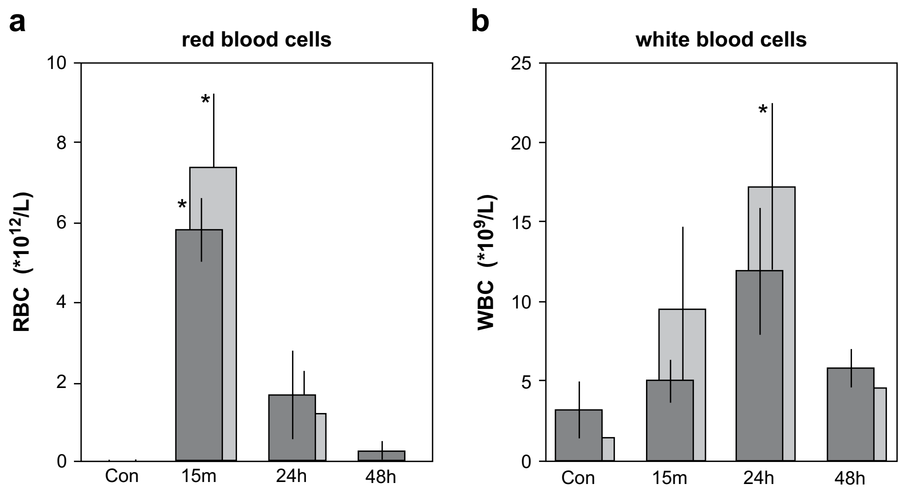

Fig. 1. RBC and WBC in the synovial fluid. Concentration of RBC (a) and WBC (b) as present in the synovial fluid, in joints without injection of blood (Con) and in the joints 15 min (15 m), 24 h (24 h) or 48 h (48 h) after blood injection. Bars in the background are from the second (verification) experiment. Mean values ± s.e.m. are given; $n = 3$. Asterisks indicate statistical significant differences ($P \leq 0.05$) compared to the control values.

decrease of 71% and 96%, respectively. Because of this rapid evacuation of RBC from the joint, this part of the study was repeated with six additional animals, providing almost identical results [bars in the background of Fig. 1(a)].

Control joints, not containing any RBC, contained about 3.2×10^9/l WBC [Fig. 1(b)], which is within the normal range of WBC in canine synovial fluid[19]. The amount of WBC measured 15 min after the injections, being 5.0×10^9/l, was within the normal range as present in whole blood of Beagles[18]. After 24 h, it was increased up to 11.9×10^9/l, but after 48 h decreased to 5.8×10^9/l, suggesting transient recruitment of WBC. These numbers were also obtained with the second set of animals [bars in the background of Fig. 1(b)].

Interestingly, there was no clear sign of clotting or lysis of the blood in the joint cavity at any time point.

DIRECT HARMFUL EFFECTS ON CARTILAGE

Despite this very quick clearance, the short presence of blood led to adverse changes in cartilage proteoglycan turnover as depicted in Fig. 2. There was significant effect of the presence of blood for 15 min on neither proteoglycan synthesis rate nor proteoglycan release. Twenty-four hours post-injection, the proteoglycan synthesis rate [Fig. 2(a)] of cartilage was decreased with 22% compared to that in control cartilage (from the uninjected knee), although not statistically significant. This inhibition in proteoglycan

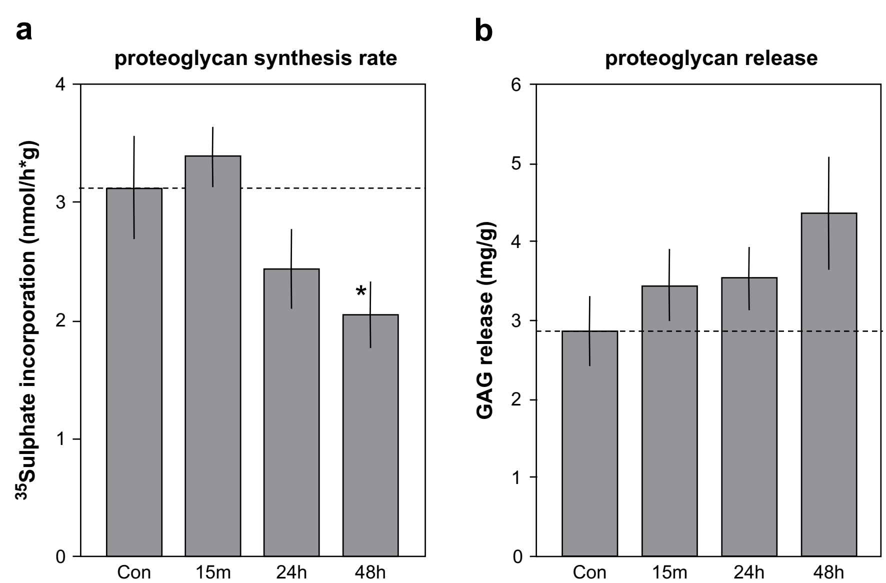

Fig. 2. Effects of intra-articular blood injections on cartilage matrix turnover. Proteoglycan synthesis rate (a) and release of proteoglycans (b) were measured 15 min (15 m), 24 h (24 h) and 48 h (48 h) after intra-articular injections of autologous blood. The control (Con) values are of cartilage of joints without injections. Mean values ± s.e.m. are given; $n = 3$. Asterisks indicate statistical significant differences ($P \leq 0.05$) compared to the control values.

synthesis rate was increased to 34% ($P \leq 0.05$) 48 h post-injection, suggesting a time-dependent process. Similarly, a time-dependent increase in release of proteoglycans [Fig. 2(b)] was observed, +24%; +53% for 24 and 48 h, respectively, although despite the magnitude and dose-dependency not statistically significant (to be expected because of the limited number of subjects studied).

SYNOVIAL TISSUE

Tissue-destructive properties of synovial tissue

Culture supernatants of synovial tissue explants of the blood-exposed joints were added to healthy canine hip cartilage to assess the effect of haemarthrosis on cartilage destructive properties of the synovial tissue. Addition of control synovial tissue culture supernatants led to the usually observed[20] decrease in proteoglycan synthesis rate [Fig. 3(a)], but this decrease almost doubled when the synovial tissue had been exposed *in vivo* to blood for 24 h or 48 h.

Addition of control supernatants or supernatants from synovial tissue obtained 15 min post-injection had no effect on the proteoglycan release of healthy cartilage [Fig. 3(b)], but the release was tripled (+200%) when supernatants of the 24 h or 48 h ($P \leq 0.05$) *in vivo* blood-exposed synovial tissues were added.

The inhibition of synthesis rate and enhancement of release resulted in a decrease of the proteoglycan content of the cartilage [Fig. 3(c)], which appeared to be

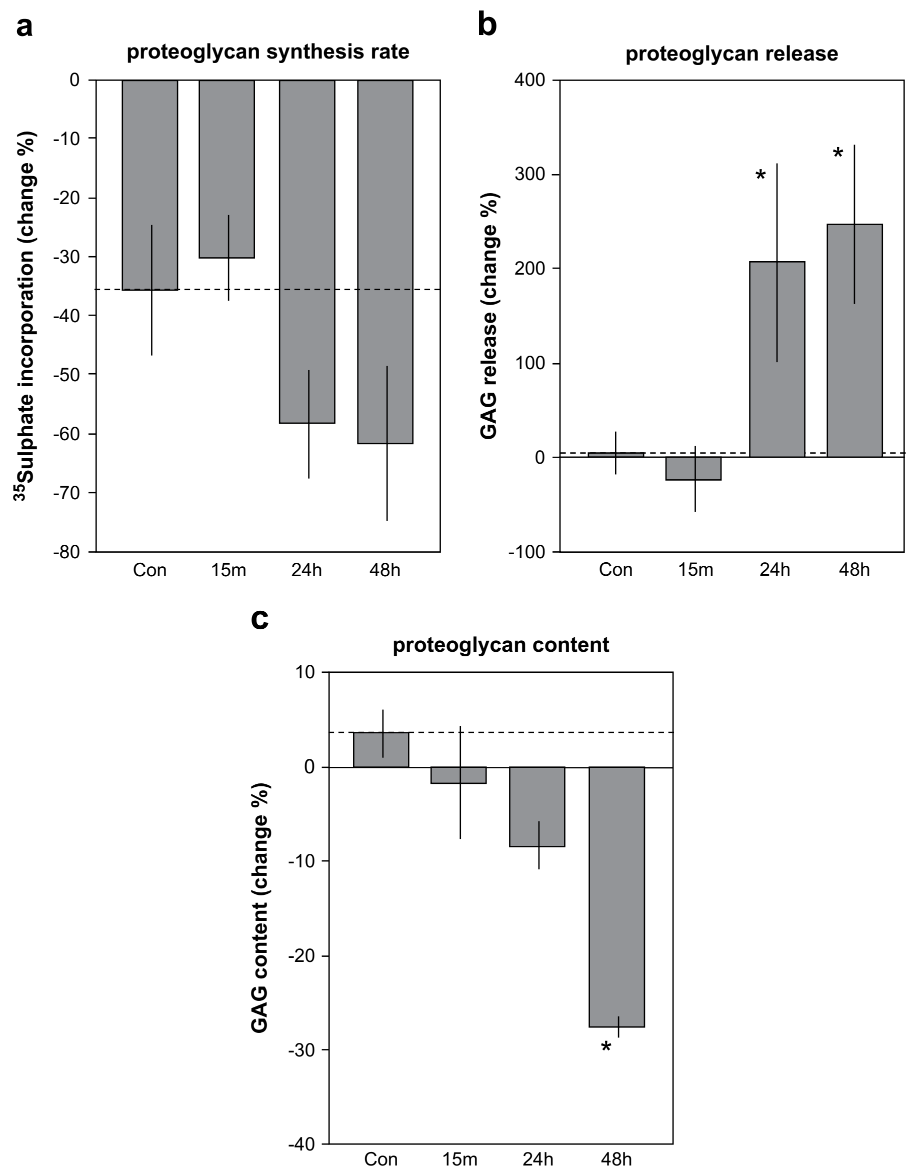

Fig. 3. Effects of synovial tissue culture supernatants on the matrix turnover of healthy cartilage. The effect of intra-articular blood on the catabolic activity of synovial tissue was measured by the effects of supernatants of these synovial tissue cultures on the matrix turnover of healthy canine hip cartilage. The proteoglycan-synthesis rate (a), -release (b), and -content (c) were measured. Synovial tissue originated from joints without blood injections (Con), or from synovial tissue 15 min (15 m), 24 h (24 h), or 48 h (48 h) after the intra-articular injections with blood. Mean values ± S.E.M. are given ($n = 3$) as a percentage compared to cartilage cultures without the addition of culture supernatants. Asterisks indicate statistical significant differences ($P \leq 0.05$) compared to the control values of uninjected joints.

time-dependent as well. A decrease of more than 25% ($P < 0.05$) was observed when culture supernatants of synovial tissue from the 48 h group were added.

Histology

The synovial tissue was stained with either HE for evaluation of general inflammatory characteristics and RBC presence, with MAC387 for monocytes/macrophages, or with Perls' Prussian Blue for iron depositions. Photographs of histological sections at the four time points after blood exposure are shown in Fig. 4(a–d).

Representative HE stained sections are depicted in Fig. 4(a and b) for the control joint and for tissue obtained 15 min after injection, respectively. Despite the synovial triggering, there was no clear sign of inflammation, as expected in such a short time period. The slightly modified[16] Goldenberg and Cohen[17] score [Fig. 5(a)] was not significantly influenced and was low compared to the maximum score of 10 that can be reached.

Striking was the enormous influx of RBC in the synovial tissue within 15 min [Figs. 4(b) and 5(b)]. The inset clearly shows numerous intact RBC in between the synovial tissue cells. These numbers of RBC decreased quickly, in parallel with the RBC count in the joint cavity [Fig. 1(a)].

The amount of MAC387-positive cells [Figs. 4(c) and 5(c)], representing monocytes/macrophages, was increased ($P \leq 0.05$) after 15 min and appeared to increase slightly in time. However, overall the amount of MAC387-positive cells was low.

The amount of Perls' Prussian Blue-positive cells was the highest after 48 h [Figs. 4(d) and 5(d)]. Nevertheless, the amount of Perls' Prussian Blue-positive cells remained very low when compared to synovial tissue of joints exposed to blood frequently as in haemophilic arthropathy[9]. A photograph representing the highest amount of Perls' Prussian Blue-positive cells observed in these samples, although still being a relatively low amount, is shown in Fig. 4(d).

Discussion

This study shows that blood is cleared very rapidly from the canine knee joint: within 48 h, the concentration of RBC in the joint cavity is decreased to less than 5%. The exact mechanism of this rapid clearance remains unsolved. Despite this quick clearance, adverse effects on both cartilage and synovial tissue are initiated.

The principal reason for this study was to elucidate the discrepancy between the severe harmful effects of blood on cartilage explants observed *in vitro* and the significantly less severe adverse effects observed in canine *in vivo* experiments. We hypothesized that an underestimation of the clearance rate could be one of the possible explanations, because rapid clearance leads to a shorter and less

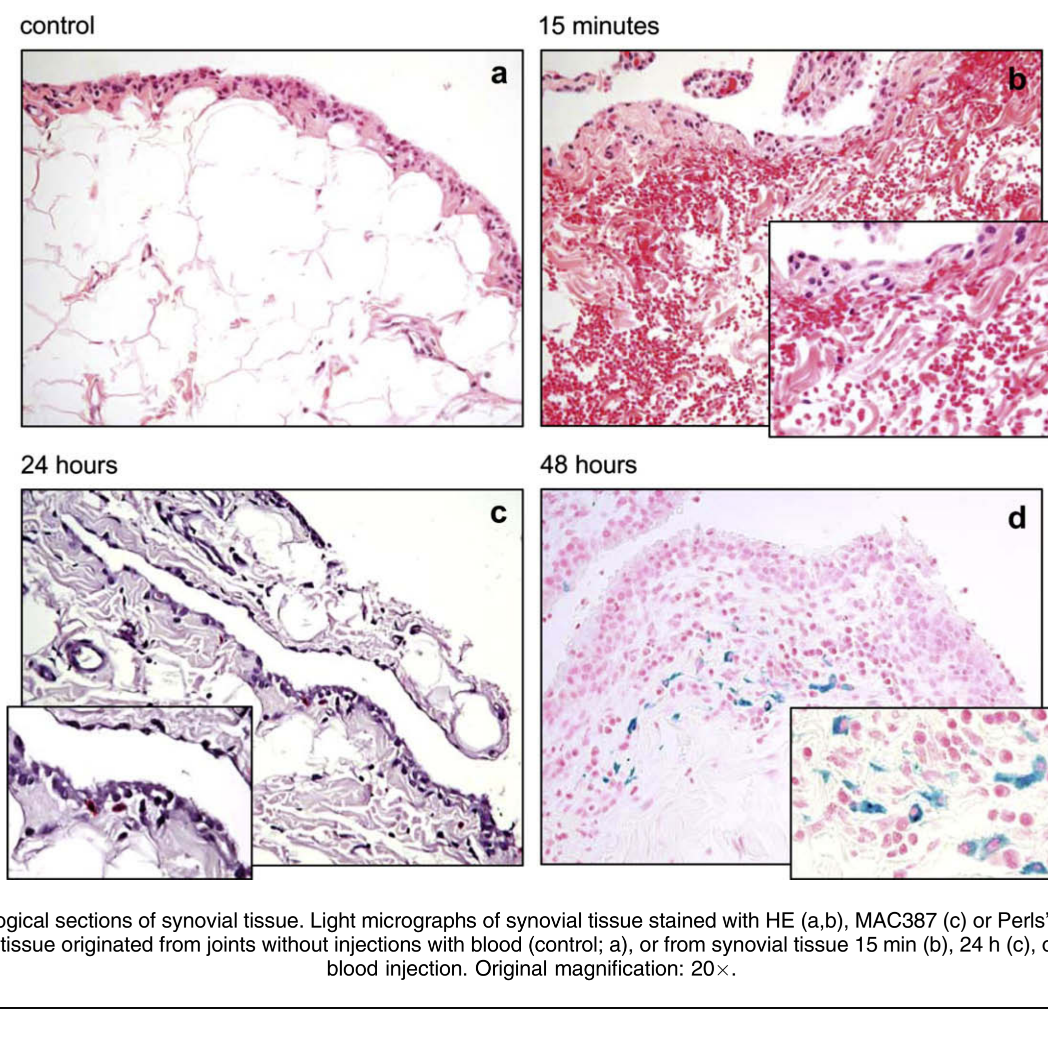

Fig. 4. Histological sections of synovial tissue. Light micrographs of synovial tissue stained with HE (a,b), MAC387 (c) or Perls' Prussian Blue (d). Synovial tissue originated from joints without injections with blood (control; a), or from synovial tissue 15 min (b), 24 h (c), or 48 h (d) after blood injection. Original magnification: 20×.

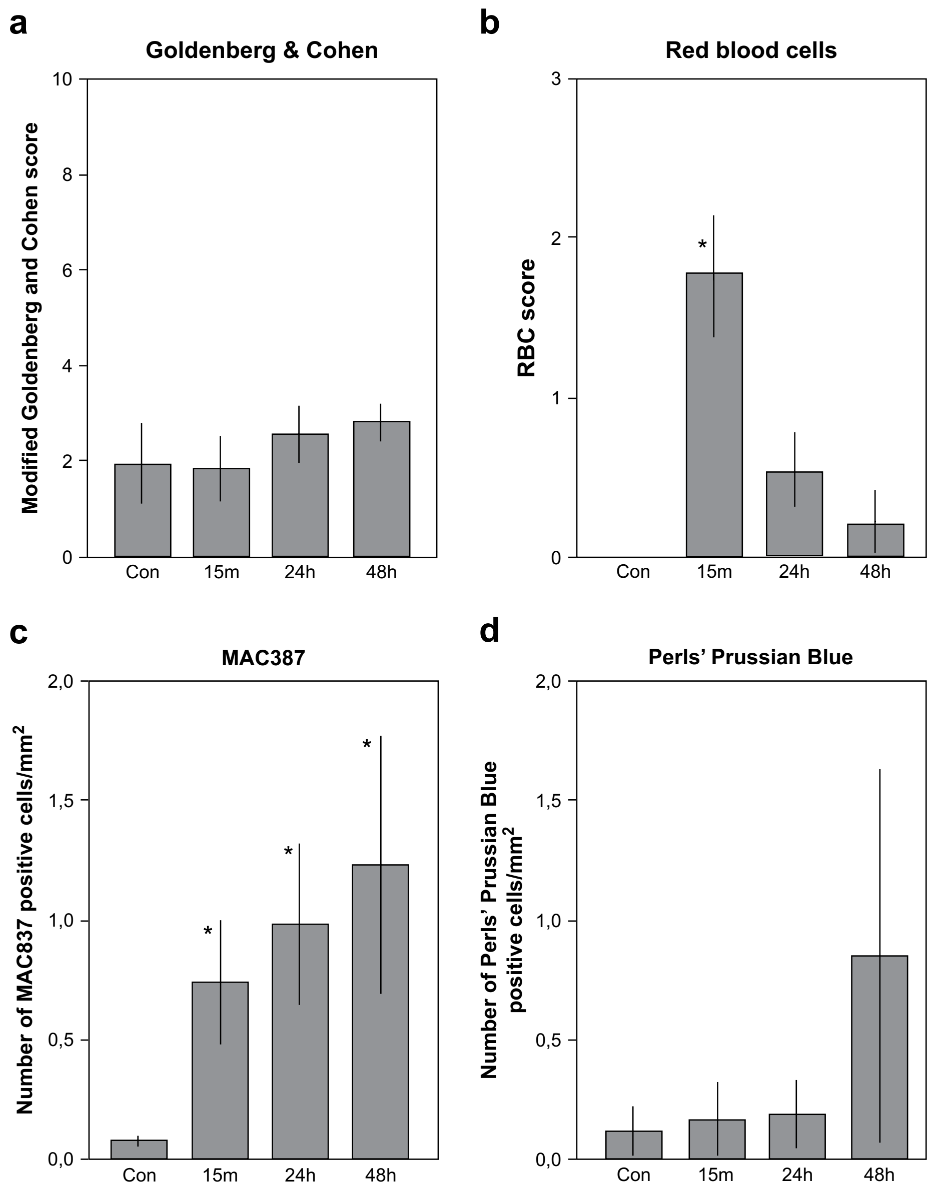

Fig. 5. Histochemical scores. (a) Modified Goldenberg and Cohen score for inflammation in HE-sections; (b) score for the presence of RBC in HE-sections; (c) number of MAC387-positive monocytes/macrophages; (d) number of Perls' Prussian Blue-positive cells for iron deposits. Synovial tissue originated from joints without blood injection (Con), or from synovial tissue 15 min (15 m), 24 hours (24 h), or 48 h (48 h) after intra-articular injection with blood. Mean values ± S.E.M. are given of $n = 6$, including the verification experiment.

intense blood exposure. Recently we showed that *in vitro* the threshold for long-lasting effects is 10% v/v blood for 48 h[11]. The present results demonstrate that *in vivo* in the canine joint the blood load is lowered below this level within this period (less than 5% in 48 h). Moreover, in the *in vitro* situation, the blood is present in a fixed amount during the whole exposure period. In the *in vivo* situation, the blood decreases gradually in time. In the *in vitro* studies a clear time- and dose-dependency of the direct harmful effects of blood on cartilage was found[11]. At the threshold blood load (10% v/v for 48 h), the effects are still long-lasting but significantly reduced to less than 50% of the harmful effects observed with 50% v/v blood for 4 days. Thus, it may be concluded that indeed rapid clearance can be (at least in part) the explanation for the observed discrepancy between the *in vitro* and *in vivo* results of blood exposure. Although the clearance rate is part of the explanation for the observed discrepancy, many other factors may be contributing *in vivo*, including inhibiting, blocking, and binding mechanisms.

Note that most of the *in vitro* experiments were conducted with homologous blood, whereas in the *in vivo* experiments autologous blood was used. However, the susceptibility of human and canine cartilage to homologous or autologous blood is similar (unpublished observations).

Of interest was the absence of significant lysis and blood clotting. An absence of blood clotting is generally recognized by aspiration of joints upon a haemarthrosis and is also observed by Valentino in his mouse model[21,22]. The limited lysis is supported by the limited number of macrophages; more abundant macrophage infiltration would be expected to clear cell debris due to significant RBC lysis.

Also the huge number of intact RBC in the synovial tissue supports the observed limited RBC lysis. Why clotting and lysis are only limited upon a haemarthrosis remains to be solved.

No reliable total volume measurements of joint fluid could be performed, relating the concentration of WBC to the exact volume of joint fluid. Thus, although not to be expected, it cannot be excluded that the temporary increase in WBC count may in part be related to a decrease in fluid volume in the joint due to selective absorption of plasma/synovial fluid.

A potential flaw in the set up of the *in vitro* experiments may be that in the *in vivo* situation, only the articular surface is exposed to blood, whereas in the *in vitro* explant system the cartilage is exposed to all sides including the cutting edges. This can also contribute to the difference in the magnitude of the effect, and thereby also to the reversibility of this effect. This issue is currently subject of study and will be published separately.

The present observation may argue the validity of the previously published *in vitro* studies. Indeed the results of these studies have to be interpreted with caution when a translation to clinical practice is discussed. Irrespectively, it is clear that a short-term exposure of cartilage to blood is harmful, not only *in vitro*, but also *in vivo*. The present study underscores this again by demonstrating the immediate significant adverse changes in both cartilage and synovial tissue. Although, based on previous canine *in vivo* research, most of these effects are not likely to be long-lasting, they can make the joint more vulnerable to a subsequent joint bleed or trauma, especially when this occurs within a period in which the joint has not fully recuperated. Furthermore, this (transiently) affected cartilage is less capable to withstand loading as we demonstrated *in vivo* before[13]. Moreover, some of the synovial changes such as iron deposition (haemosiderosis) may not be reversible and a permanent trigger for inflammatory activity.

A question that raised from the present study is how the quick clearance of blood from the joint is accomplished. Striking was the fact that the synovial tissue of the joints were loaded with RBC already 15 min after the haemarthrosis was induced, but that this amount of RBC was far less already after 24 h and that almost no RBC were found in the synovial tissue 48 h after injection. It has been suggested that RBC can be opsonised when entering the joint cavity and can be recognized as foreign by macrophages and synoviocytes[23]. However, although there was an increase in monocytes/macrophages, this increase was not of a magnitude expected to be able to remove this load of RBC.

Interestingly, also the degree of Perls' Prussian Blue staining was limited, certainly compared to the amount in synovial tissue of joints exposed to blood more frequently as in haemophilic arthropathy[9], suggesting only a limited deposition of iron in the form of haemosiderin in the synovial tissue. Although it is difficult to estimate how many RBC are needed to deposit sufficient iron-containing material to get the intensity and number of Perls'-positive cells as observed, it can hardly be that the increase in Perls'-staining in this study includes the iron from all the RBC cleared from the joint cavity and synovial tissue. In that case, it would have been more likely that a broader distribution of Perls'-staining was found. This suggests that rapid degradation is not taking place on a large scale. Apparently, other mechanisms of clearance must take place.

A possibility is that intact RBC re-enter the peripheral blood directly or *via* the lymphatic system. However, we can only speculate on this, for there is no clear evidence of the exact process. In future studies it would be interesting to label the blood cells before intra-articular injection to trace the route by which the cells are cleared from the joint cavity after a joint haemorrhage.

Previous clearance studies in rabbits[24,25] have shown a less quick clearance, varying from one- to two-third of the injected amount within 1 week. The contradiction with our quick clearance could originate from the limited discriminating ability of the technique used; the decrease in radioactivity signal upon injection of ^{59}Fe-labelled RBC was measured outside the joint using a probe, giving no information of the exact location of the radiolabeled RBC (synovial fluid, synovial tissue, or even peri-articular). Some tissue analysis was performed, however, on most time points only from one animal, making it neither able for extensive analysis and conclusions, nor for comparison with the present study. On the other hand, the extreme clearance rate in our study could be specific for the canine species.

Irrespectively, the rabbit study and clinical practice with haemarthroses in haemophiliacs were the premises for the estimation in our previous studies that 4 days 50% v/v blood mimics the blood load during a joint haemorrhage. It may indeed be argued (and needs to be studied) whether this extreme rapid clearance also occurs in the human joint, as clinical practice suggests differently. Based on clinical practice in the treatment of haemophilia patients (personal observation; GR), it is not likely that in humans the blood load is decreased below 10–20% within 2 days, meaning that in humans a single joint haemorrhage probably has more serious direct adverse effects, with a more long-lasting character, and predisposes for damage from subsequent influences such as load and repeated bleeding.

Taken together, rapid clearance of blood from the canine knee joint contributes to the discrepancy between the long-term *in vitro* and *in vivo* effects of blood-induced joint damage. More research is needed to fully elucidate the mechanisms of clearance. Irrespectively, blood has devastating effects on articular cartilage very rapidly, both directly and *via* the synovial tissue, and in this respect it remains important to prevent (traumatic) joint haemorrhages and if they occur, to treat them properly.

Conflict of interest

The authors declare that they have no conflict of interest.

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